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Blocking porcine sialoadhesin improves extracorporeal porcine liver xenoperfusion with human blood

Joshua P. Waldman[*,1], **Thomas Vogel**[*,2], **Christopher Burlak**[3], **Constantin Coussios**[4], **Javier Dominguez**[5], **Peter Friend**[6], and **Michael A. Rees**[1]

[1]Department of Urology, University of Toledo Medical Center, Toledo, OH, USA

[2]Department of General and Visceral Surgery, University Hospital Münster, Münster, Germany

[3]Department of Surgery, Indiana University School of Medicine, IU Health Transplant Institute, Indianapolis, IN, USA

[4]Institute of Biomedical Engineering, Department of Engineering Science, Old Road Campus Research Building, University of Oxford, Headington, Oxford, OX3 7DQ, United Kingdom

[5]Dpto. Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA),28040, Madrid, Spain

[6]Nuffield Department of Surgical Sciences, University of Oxford, Oxford Transplant Centre, OX3 7LJ, United Kingdom

Abstract

Patients in fulminant hepatic failure currently do not have a temporary means of support while awaiting liver transplantation. A potential therapeutic approach for such patients is the use of extracorporeal perfusion with porcine livers as a form of “liver dialysis”. During a 72-hour extracorporeal perfusion of porcine livers with human blood, porcine Kupffer cells bind to and phagocytose human red blood cells (hRBC) causing the hematocrit to decrease to 2.5% of the original value. Our laboratory has identified porcine sialoadhesin expressed on Kupffer cells as the lectin responsible for binding N-acetylneuraminic acid on the surface of the hRBC. We evaluated whether blocking porcine sialoadhesin prevents the recognition and subsequent destruction of hRBCs seen during extracorporeal porcine liver xenoperfusion. Ex vivo studies were performed using wild type pig livers perfused with isolated hRBCs for 72-hours in the presence of an anti-porcine sialoadhesin antibody or isotype control. The addition of an anti-porcine sialoadhesin antibody to an extracorporeal porcine liver xenoperfusion model reduces the loss of hRBC over a 72 hour period. Sustained liver function was demonstrated throughout the perfusion. This study illustrates the role of sialoadhesin in mediating the destruction of hRBCs in an extracorporeal porcine liver xenoperfusion model.

Address correspondence to: Michael Rees, University of Toledo Medical Center, Department of Urology, Mail Stop 1091, 3000 Arlington Avenue, Toledo, OH 43614, Office 419-383-6774, Michael.rees2@utoledo.edu.

*These authors contributed equally to this study

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Keywords

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INTRODUCTION

Xenotransplantation provides the hope for an unlimited supply of organs available for patients awaiting organ transplantation. A potential application within the field of xenotransplantation is the use of a porcine liver in an extracorporeal support device to serve as a bridge therapy for patients in fulminant hepatic failure (FHF). Given that the liver is known to be resistant to complement-mediated rejection [1-3], it is not surprising that modifications to overcome complement-mediated rejection have not improved the performance of extracorporeal porcine liver perfusion (ECLP) to the point that it is ready for clinical introduction. Thus, alternative obstacles involving the direct recognition and destruction of xenogeneic cells have been sought to understand the barriers that limit the clinical application of ECLP. Previously, we and others have shown that porcine livers perfused with human blood mount several forms of graft versus host “reactions”. Within only 15 minutes of perfusion, porcine livers consume as many as 10^{11} human platelets [4, 5]. Additionally, our laboratory has shown that porcine livers perfused with whole human blood consume the equivalent of 3 units of human erythrocytes over a 72-hour period [6]. The consumption of human platelets and erythrocytes by the porcine liver decreases the ability of the liver to maintain metabolic parameters in the extracorporeal perfusion system. Before extracorporeal porcine liver perfusion can be realized as a viable treatment for patients in FHF, these graft-vs.-host “responses” must be addressed.

Our laboratory has identified the involvement of porcine Kupffer cells (KC) in mediating the clearance of human red blood cells (hRBC) by porcine livers in an extracorporeal perfusion model [7]. Previous studies demonstrated that the loss of hRBCs was not due to antibody-mediated destruction and did not involve complement opsonization of the xenogeneic erythrocytes [8]. Rather, the clearance of hRBCs resulted from a direct recognition of hRBCs by porcine macrophages [7]. Burlak et al identified N-acetylneuraminic acid (Neu5Ac) on the surface of the human erythrocyte as the sugar involved in mediating the interaction between human erythrocytes and the porcine macrophages [9]. Brock et al. subsequently identified porcine sialoadhesin (pSn) expressed on the surface of the porcine KC as the lectin responsible for binding Neu5Ac on the surface of the hRBC [10].

Sialoadhesin, a highly conserved protein expressed in mammals, was originally identified as a sheep erythrocyte receptor and is known to bind the Neu5Ac form of sialic acid [11-14]. Additionally, sialoadhesin has been shown to play a role in various immune functions including antigen presentation and internalization of viruses like porcine reproductive and respiratory syndrome virus and human immunodeficiency virus [15-17]. Knockout studies performed in mice suggest that sialoadhesin may also play a role in maintaining T cell and B cell population distribution in the spleen and lymph node, as well as facilitating immunoglobulin M production[18]. Given that anti-pSn monoclonal antibody (mAb)

prevents the in vitro recognition of the hRBCs by porcine macrophages, we wished to test if anti-pSn mAb would prevent the loss of human erythrocytes during extracorporeal porcine liver xenoperfusion [10]. We observed that addition of anti-pSn mAb during extracorporeal porcine liver xenoperfusion reduced hRBC clearance and maintained hepatic function over a 72-hour period.

MATERIALS AND METHODS

In vitro erythrocyte binding assay

The ability of porcine macrophages to bind human erythrocytes in the presence of a mouse IgG_{2a}-anti-pSn mAb, 1F1, previously described by Revilla et al. [19], or an isotype control, was analyzed using an *in vitro* binding assay. 1F1 was chosen in part because this antibody binds to the carbohydrate-binding domain of porcine sialoadhesin (unpublished data). Porcine macrophages isolated from the lung as described by Wensvoort et al, were cultured for three days and then seeded into 96-well round bottom plates at 30×10^3 cells per well [20]. Porcine alveolar and Kupffer cell macrophages were used interchangeably for in vitro experiments as previously demonstrated by Brock et al [10]. Cells were then treated with 1F1 mAb or an isotype control Ab for 1 hour after which the RPMI-1640 media (Sigma-Aldrich, St. Louis, MO) was removed and human erythrocytes were added. 1F1/isotype control mAb and hRBCs were diluted with RPMI at concentrations of 1 and 10μg/ml of 1F1 or isotype control and 0.1% packed hRBCs. Macrophages were co-incubated with erythrocytes for 2 hours upon which time wells were washed with RPMI to remove unbound erythrocytes. Cells were then fixed with 100% methanol and bound hRBCs were quantified using the tetramethylbenzidine (TMB) reaction. Plates were reacted and then quantified using a spectrophotometer at the 450nm wave length. Data were calculated as percent binding, relative to non-treated porcine macrophages co-incubated with human erythrocytes.

Determining amount of 1F1 mAb needed in ex vivo perfusion

In vitro and ex vivo techniques were utilized in order to determine the concentration of 1F1 mAb needed to block pSn in the ex vivo perfusion model. As described above, we performed a series of in vitro sighting assays wherein cultured porcine macrophages were incubated with the 1F1 blocking antibody in increasing concentrations and subsequently exposed to human erythrocytes. To calculate the amount of mAb needing to block all pSn molecules expressed in the liver, we calculated the amount of mAb needed to block erythrocyte binding of one macrophage. Based on our in vitro data where 100 μl of a 10μg/ml solution of 1F1 mAb saturated the pSn receptors of 3×10^4 porcine macrophages (see Fig. 1B), we determined that 0.03ng of 1F1 mAb was needed to block the erythrocyte binding of one macrophage. Using the estimate of Bouwens et al., which estimated 4.1×10^7 to 1×10^8 KC in 100 grams of rat liver, we calculated the expected number of KC in a 1200g porcine liver as being 4.9×10^8 and 1.2×10^9 KC [21]. Taken together with the amount of mAb needed to block erythrocyte binding of one macrophage, we estimated that 14-30 mg of the 1F1 mAb would achieve complete saturation of all pSn sites.

In order to account for the kinetics of 1F1 mAb in the ex vivo perfusion model, given flow, time, possible binding and internalization, and the de novo expression of new pSn, we

performed a single sighting experiment to determine the calculated amount of mAb needed to block sialoadhesin expressed in the ex vivo porcine liver. We aimed to achieve a concentration of approximately 10μg/ml, consistent with our in vitro inhibition data. Using the liver perfusion method by Butler et al., a porcine liver was perfused with porcine blood and 1F1 mAb was added to the perfusate every hour in 5mg increments starting with 0mg at time zero and finishing with 40mg at 8 hours of perfusion [6]. Serum samples and liver biopsies were collected every half hour prior to 1F1 mAb injection, flash frozen, and later analyzed for 1F1 mAb serum concentration using an enzyme-linked immunosorbant assay (ELISA) and also for 1F1 mAb binding in the liver by immunohistochemistry.

Blood and liver donors

The methods used in this study have been published previously and are described here briefly [6]. Human packed red blood cells were donated by the United Kingdom National Health Service blood bank. Blood was from group O patients and was leukocyte depleted prior to use. Blood was fully heparinized immediately prior to use, calcium levels were corrected by the addition of calcium gluconate. Large white pigs (50-60 kg) were used in all experiments. All animals were treated in accordance with the Animals (Scientific Procedures) Act, 1986.

Removal and preparation of porcine livers

Liver donors were intubated and ventilated. Anesthesia was maintained with inhalative isoflurane. The liver was mobilized until connected only by its vascular attachments. After systemic heparinization, a cannula was placed into the distal aorta and 3 liters of cold Soltran (Baxter Healthcare, Deerfield, IL) commenced after aortic cross clamping. Following hepatectomy, the infrahapatic inferior vena cava (IVC), portal vein, and the hepatic artery were cannulated. The bile duct was cannulated and bile output was measured during perfusion. The final 500 ml of cold Soltran infused into the liver contained 12 mg of 1F1 or isotype control Ab and cold ischemic time was approximately 2 hours before initiating perfusion.

Extracorporeal liver perfusion

Six separate normothermic extracorporeal liver perfusions, 3 with an anti-Sn mAb (1F1) and 3 with the isotype control. The liver was perfused according to the method of Butler et al. using a prototype of the OrganOx perfusion device [6, 22]. In short, the perfusion circuit, consisting of a blood reservoir, an oxygenator, a heating element, and a centrifugal pump,was primed with crystalloid solution (sterofundin®, B.Braun) upon which time packed leukocyte depleted red blood cells were added until hematocrit reached normal physiologic range. At this time pH, p_aCO_2, p_aO_2 and Ca^{2+} were adjusted to normal physiologic range. Before connection of the liver to the perfusion circuit, the preservation fluid was flushed from the liver using 1L of crystalloid infusion solution at room temperature. The liver was then connected to the primed perfusion apparatus. Prostacyclin, heparin, and total parenteral nutrition (TPN) were infused throughout the perfusion. Additionally, glucose and insulin were given in order to maintain normal glucose levels. Cefotaxime was added at the beginning of the perfusion and every 24 hours thereafter. Bile

acids were replaced with taurocholic acid (New Zealand Pharmaceuticals,NZ) at 140 mg/hour.

Consistent with our data from the ex vivo sighting experiment, we administered either the 1F1 or the isotype control with the goal of maintaining a concentration of 10μg/ml throughout the duration of the perfusion. In order to achieve the desired concentration of antibody and block pSn prior to exposure of the pig livers to hRBC, we chose the following method to administer 1F1 mAb and the isotype control Ab during porcine liver xenoperfusion: 1) 12 mg of antibody was infused into the liver immediately following procurement and left in the preservation solution in the liver for approximately two hours; 2) 12 mg of antibody was infused into the human blood perfusate prior to connection to the pig liver; 3) 12 mg of antibody was infused after 24 hours and again after 48 hours of perfusion. In this way, we expected to achieve saturation of pSn receptors prior to exposure of the pig livers to hRBC as well as allowing for replacement of phagocytosed 1F1 mAb and blockage of new pSn synthesized and expressed over the course of the perfusion with subsequent bolus administration. Discussions with pharmaceutical company scientists with expertise in determining appropriate doses of mAb for clinical trial determined this approach was reasonable given the time and budgetary constraints of these ex vivo perfusion experiments that cost over $5,000/perfusion.

Collection of blood and tissue samples

Blood samples were obtained immediately prior to cross circulation of liver (t=−1), immediately following cross circulation (t=0), and every 2 hours thereafter for the remainder of the perfusion. Blood gas analysis was performed immediately. Blood samples were later analyzed for full blood count, urea, electrolytes, creatinine, and biochemical liver function tests. Plasma was collected, flash frozen in liquid nitrogen and stored for later analysis.

Microscopy

Biopsies were harvested from the periphery of the right lobe at time of back table cannulation (t=−2hrs), at 24hrs, 48hrs and preserved in either 10% formalin or flash frozen in optimal cutting temperature (OCT) media (Electron Microscopy Sciences, Hatfield, PA). Complete liver dissection was performed at the termination of perfusion and samples were prepared for both immunohistochemical analysis and Hematoxylin and Eosin staining. Determining co-localization of mouse antibody with sinusoidal walls was performed by labeling frozen sections fixed with 4% paraformaldehyde and blocked with 50% odyssey blocking buffer (LI-COR, Lincoln, NB) in phosphate-buffered saline (PBS) with 1μg/ml goat anti-porcine CD31 antibody (R&D Systems, Minneapolis, MN). Goat antibody was detected with DyLight 549 labeled rabbit anti-goat IgG (H&L) antibody (0.2μg/ml) (Jackson ImmunoResearch Laboratories, West Grove, PA). In order to identify 1F1 mAb or the isotype control Ab, tissue sections were labeled with DyLight 649 labeled Donkey anti-mouse IgG antibody (0.2μg/ml) (Jackson ImmunoResearch Laboratories, West Grove, PA). Tissue sections were washed using blocking buffer containing 0.1% tween 20. Nuclei were stained using DAPI (Life Technologies, Carlsbad, CA) (1:5000 in PBS) for 1 min.

Flow Cytometry

Blood was collected from healthy individuals and red blood cells, leukocytes, and lymphocytes were isolated. Porcine macrophages were isolated according to Wensvoot et al [20] and used as a positive control. Isolated cells were suspended in blocking buffer (phosphate-buffered saline (PBS) containing 20% horse serum) and incubated on ice for 30 minutes in PBS and then stained for 30 minutes at 4°C with 1F1 mAb or the isotype control Ab at a concentration 0.9μg/ml diluted in blocking buffer. Cells were washed and then stained with 0.1μg/ml ALEXAFLOUR-488 conjugated goat anti-mouse IgG secondary (Invitrogen, Carlsbad, CA) at 4°C for 30 minutes in order to detect primary antibody binding. Individual cell populations were analyzed for 1F1 mAb or isotype control Ab binding via flow cytometry.

Western Blot Analysis

To determine 1F1 mAb reactivity with pSn, porcine macrophage homogenates were prepared and protein was separated on 6% SDS-PAGE gel. Protein was transferred to a nitro-cellulose membrane, blocked with blocking buffer (PBS containing 0.5% nonfat dried milk). Membranes were then incubated with 1μg/ml of 1F1 mAb diluted in blocking buffer. As a loading control, membranes were probed with 0.2μg/ml mouse anti-human α-tubulin (Santa Cruz, Santa Cruz, CA). Primary antibody binding was detected using horse radish peroxidase (HRP) conjugated goat-anti-mouse IgG secondary antibody (Southern biotech, Birmingham, AL) at a concentration of 0.25μg/ml in blocking buffer for 2 hours at room temperature. Membranes were developed using an ECL western blot analysis kit (GE Healthcare, Waukesha, WI). To detect soluble pSn in the perfusion circuit, serum samples collected from perfusions were first diluted 1:100 in PBS and separated via electrophoresis. Protein was transferred to a nitrocellulose membrane. Membranes were blocked with blocking buffer for 2 hours at room temperature. Membranes were then probed using 1F1 mAb at a concentration of 1μg/ml of 1F1 mAb in blocking buffer at 4°C overnight. 1F1 mAb binding was detected as described above.

ELISA

High protein binding 96-well plates (Corning Incorporated, Corning, NY) were incubated with 10μg/ml of goat anti-mouse IgG_{2a} antibody (Jackson Immunoresearch Laboratories, West Grove, PA) in coating buffer for 4 hours at room temperature and then blocked overnight with blocking buffer (PBS containing 1% BSA, 1% casein and 0.5% lysine) at 4°C. After washing, capture antibody was incubated with plasma serially diluted in ELISA diluent for 45 minutes at room temperature. Captured 1F1 mAb, or the isotype control Ab, was detected with HRP conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) at 1:5000 in ELISA diluent by incubation for 45 min at room temperature. Plates were developed using OPD solution (dH_2O containing o-phenylenediamine) and were analyzed with a spectrophotometer.

Statistical Analysis

Data were analyzed with SPSS Statistics 17.0 using Student's *t*-tests and repeated measures ANOVA. p-Values of ≥0.05 were considered statistically significant.

RESULTS

1F1 mAb inhibits pSn mediated binding of human erythrocytes in vitro

First, we set out to determine whether porcine macrophage-mediated human erythrocyte binding could be inhibited using the mAb, 1F1, as an alternative to the anti-pSn mAb, 41D3, previously described by Brock et al. The mAb 1F1 was chosen based on its specificity for the carbohydrate-binding domain of pSn. Binding of 1F1 mAb to pSn on isolated porcine macrophages was demonstrated by flow cytometry (Fig. 1A. i). Western blot analysis showed a single band of about 185KDa, the expected size of pSn (Fig 1A. ii). An in vitro erythrocyte binding assay was performed to determine the ability of 1F1 mAb to inhibit rosette formation. We found that 1μg/ml of 1F1 mAb inhibited rosette formation by nearly 60% of that seen when hRBCs are exposed to untreated macrophages ($p<0.01$), while 10μg/ml of 1F1 mAb blocked rosette formation by 95% ($p<0.001$), levels not significantly different from porcine erythrocyte binding (Fig. 1B). Microscopic images of porcine macrophages pretreated with either 10μg/ml of 1F1 mAb or the isotype control Ab and subsequently co-incubated with human erythrocytes verify quantitative analysis of rosette formation (Fig. 1C).

Determining amount of 1F1 mAb required to block pSn during extracorporeal liver perfusion

In a single ECLP sighting experiment, we aimed to achieve a concentration of 10μg/ml of 1F1 in the perfusate consistent with the concentration producing 95% inhibition of porcine KC binding of the human RBC in vitro (Fig. 1B) As described in the section, “Determining the amount of 1F1 needed in the ex vivo perfusion”, we performed an ex vivo sighting experiment wherein an increasing amount of 1F1 was added to the perfusate of an extracorporeal porcine liver perfusion in increments of 5mg. As seen in figure 2A, we observed that upon adding 25mg of antibody, the concentration of 1F1 in the perfusate had reached approximately 10μg/ml (Fig. 2A). Fluorescence microscopy images of the liver taken at times following injection of 1F1 suggests that 1F1 saturation of pSn binding sites on Kupffer cells in the liver was achieved after 25mg of 1F1 had been added to the perfusate (Fig. 2B). We base this on the fact that our data demonstrates no additional binding in the sections that we reviewed after 25 mg had been added. In fact, while 25, 30 and 35 mg all demonstrated a similar amount of binding (and greater binding than any of the smaller concentrations), the binding with 40 mg was actually less than that seen after 25 mg had been given. Taken together, we determined that a concentration of approximately 10μg/ml of 1F1 mAb would result in consistent inhibition of pSn and that this amount could be achieved by the infusion of approximately 25 mg of 1F1 mAb into the perfusion apparatus (Fig. 1B and Fig. 2).

Normal hemodynamic and synthetic function in livers treated with 1F1 mAb

In order to determine the effect of the addition of 1F1 mAb on liver function, hemodynamic and synthetic parameters were assessed. In all of the perfusions, portal flow and pressure, as well as IVC flow and pressure, remained consistent and were not significantly different (Fig. 3A). Additionally, continuous production of albumin, urea and bile were demonstrated in the 1F1 mAb and isotype control Ab treated groups with no differences observed (Fig. 3B).

Finally, we examined alanine transaminase levels and found that in both the 1F1 mAb treated and isotype control Ab treated, terminal ALT levels were within the normal range for the pig and were not significantly different (Fig. 3B).

The addition of 1F1 mAb prolongs metabolic function

In order to investigate the metabolic effect of blocking pSn, and presumably reducing erythrocyte destruction, on xenogeneic liver perfusion, metabolic function was assessed by measuring pH, bicarbonate, and base excess throughout the perfusion of 1F1 mAb and isotype control Ab treated livers. Although progressive acidosis was observed in both 1F1 mAb and isotype control Ab treated livers beginning at 48 hours, acid-base function was markedly impaired in the isotype control Ab treated group as compared to those livers treated with 1F1 mAb (p=0.003) (Fig. 4A). Additionally, oxygen consumption (ml/min) by each liver was measured throughout the perfusion using the Fick principle (Fig. 4B). A consistent volume of oxygen was consumed throughout perfusion in those livers treated with 1F1 mAb. However, continuous decrease in oxygen consumption over time was seen in those livers treated with the isotype control Ab. The progressive decrease in oxygen consumption seen in isotype control Ab treated livers resulted in a significant difference in oxygen consumed by isotype control Ab treated livers as compared to those livers treated with 1F1 mAb beginning at 54 hours of perfusion (P=0.003) (Fig. 4B).

Kupffer cell mediated erythrocyte destruction reduced by 1F1 mAb

In order to determine the effect of 1F1 mAb on Kupffer cell-mediated destruction of hRBCs by porcine livers, perfusate samples were obtained throughout the perfusion and analyzed for the presence of intact red blood cells present (hematocrit). As shown in Figure 5A, there was a time dependent decrease of the hematocrit in livers treated with both the isotype control Ab and 1F1 mAb (P<0.001 and P<0.001, *respectively*). Analysis of the hematocrits of the 1F1 mAb versus isotype control Ab treated livers revealed a greater loss of hematocrit in the perfusion of those livers treated with the isotype control Ab as compared to livers treated with 1F1 mAb (P=0.01) (Fig. 5A). Levels of circulating mouse Ab in the serum of livers treated with anti-pSn antibody or an isotype control Ab were determined by an enzyme-linked immunosorbant assay. We found that though equal amounts of 1F1 mAb and isotype control Ab were administered, circulating isotype control Ab levels reached a concentration of 15.95μg/ml whereas the 1F1 mAb concentration reached a maximum of only 5.1μg/ml (P<0.01) (Fig. 5B). H&E analysis was performed on 1F1 and isotype treated livers and representative images are shown in figure 5C.

1F1 mAb localizes to the liver

In order to determine if decreased erythrocyte destruction was the result of 1F1 mAb binding to pSn in the liver, we biopsied livers throughout the perfusion and subsequently performed confocal microscopy to evaluate the amount of mouse antibody binding. We found livers treated with the 1F1 mAb had a considerable amount of mouse IgG deposition, whereas those treated with the isotype control Ab did not (Fig. 6A). This suggests that inhibition of erythrocyte destruction was the result of 1F1 mAb binding in the liver and that the difference observed in the concentration of 1F1 mAb and the isotype control Ab found in

the perfusion circuit was the result of 1F1 mAb being bound in the liver. Next we determined whether the liver was the only place where 1F1 mAb was being sequestered. We reasoned that other potential sites for 1F1 mAb sequestration in this model were limited primarily to cells in the perfusate and free floating pSn potentially solubilized in the serum. In order to test if 1F1 mAb was being bound by cells in the perfusate, we analyzed 1F1 mAb binding capabilities of human erythrocytes, platelets, lymphocytes, and granulocytes; 1F1 mAb was not bound to a greater extent than the isotype control Ab in any human cell examined (Fig. 6B). Finally, in order to determine if soluble pSn was present in the serum of the perfusate, we performed western blot analysis on serum samples isolated throughout the perfusion using 1F1 mAb as the primary Ab. No detectable pSn was observed using this method (Fig. 6C). These data suggest that the porcine liver was the primary binding region of 1F1 mAb.

DISCUSSION

In the present study, we demonstrate the ability of an anti-pSn mAb to inhibit the destruction of human erythrocytes during extracorporeal porcine liver xenoperfusion. While pSn inhibition does not completely eliminate destruction of human erythrocytes by porcine Kupffer cells, it is likely that further refinement in the dose of antibody given and the administration strategy utilized, would optimize the inhibition observed. Thus, the current findings highlight the role of innate cellular carbohydrate recognition by the lectin, porcine sialoadhesin, in mediating the process underlying this xenogeneic graft vs. host response. Furthermore, we demonstrate the maintenance of liver function in an extracorporeal xenoperfused liver for a 72 hour duration, with improved function observed in those livers treated with the anti-pSn mAb, 1F1.

The use of a porcine liver in an extracorporeal support device for patients in FHF has the potential of serving as either a bridge to allotransplantation or to recovery, thereby avoiding the need for transplantation. However, clinical application is limited by several forms of graft-vs-host reactions directed toward components of human blood [6]. Initial experiments demonstrated that both wild type pig livers and hDAF transgenic pig livers perfused with human blood consumed nearly all of the human platelets within minutes of initiating perfusion, and over three days of perfusion destroyed the majority of human erythrocytes, with hDAF transgenic livers causing greater erythrocyte loss than wild type [23]. Thus, while the experiments described here utilized wild type pig livers, use of genetically modified pig livers causes more rapid loss of human erythrocytes during xenoperfusion, so that future experiments testing pSn inhibition in hDAF transgenic/GTKO pig livers is warranted.

Since the initial experiments that identified the graft-vs.-host anti-erythrocyte xenogeneic response under study, a mechanism responsible for the recognition of human erythrocytes by porcine Kupffer cells has been identified. Human erythrocyte removal by porcine Kupffer Cells appears to be mediated through a mechanism involving surface expressed sialoadhesin binding to Neu5Ac on the surface of human erythrocytes [9, 10]. Data published in the glycol-biology field suggests the presence of a human specific deletion in CMP-Neu5Ac Hydroxylase, the gene responsible for converting Neu5Ac to N-Glycolylneuraminic acid

[24, 25], and that humans are the only primate species that expresses the majority of its sialic acid in the Neu5Ac form [26]. Taken together, it is possible that humans will be the only species whose red blood cells are consumed by porcine Kupffer cells in a pSn dependant mechanism. The current data extends these observations by demonstrating that mAb 1F1, with known specificity for the Neu5Ac binding domain of pSn (unpublished data), not only inhibits porcine macrophage binding of hRBC in vitro (Fig. 1), but also reduced human erythrocyte destruction during extracorporeal porcine liver xenoperfusion (Fig. 5).

The observation that 1F1 mAb did not completely prevent human erythrocyte loss in the ex vivo perfusion model could have resulted for several reasons. It is possible that the incomplete blockage of erythrocyte destruction was the result of inadequate concentrations of 1F1 mAb in the perfusion circuit. It is worth noting that the lowest rates of erythrocyte loss throughout the perfusion occurred at times immediately following injections of 1F1 mAb; times during the perfusion when mAb concentrations were highest (Fig. 4A and 4B). Prior in vitro work from our laboratory suggested that a concentration of 10μg/ml of 1F1 mAb resulted in 95% inhibiting of human erythrocyte binding by porcine macrophages (Fig. 1). As seen in Figure 2, an ex vivo sighting experiment performed in our laboratory suggested that the addition of 25mg of 1F1 mAb would result in a circulating concentration near 10μg/ml. It was expected that an additional 12mg of mAb added at times 24hrs and again 48hrs after commencement would counteract a decrease in circulating 1F1 mAb resulting from pSn binding and internalization by porcine KCs over the course of the perfusion. Despite efforts to maintain 10μg/ml of 1F1 mAb in the perfusate, ELISA assays performed on perfusate serum collected throughout the present study illustrated that 1F1 mAb concentrations reached a maximum of 5.1μg/ml while the concentration of the isotype control Ab reached concentrations of 15.9μg/ml. Thus, it is possible that insufficient 1F1 mAb was present to achieve maximal inhibition. Further studies could be performed to determine a more optimal dose of 1F1 mAb, but as our purpose was to determine whether pSn inhibition would reduce hRBC destruction, these experiments have served their purpose. Rather than further experiments to refine the optimal dose of 1F1 mAb, work is in progress to produce a sialoadhesin knock out pig as a definitive strategy.

In addition to the possibility of inadequate dosing of 1F1 mAb, the discrepancy between in vitro and ex vivo inhibition may be explained by antibody-mediated receptor endocytosis triggered by antibody binding to pSn removing antibody from the circulation [19, 27]. Alternatively, it is possible that 1F1 mAb binding to circulating cells or proteins altered the circulating concentration of 1F1 mAb. However, flow cytometric analysis of cells expected to be found in the perfusate showed no binding of 1F1 mAb and western blot analysis of serum isolated from the perfusate revealed no appreciable amount of pSn.

Data from this study may also be evidence that additional recognition pathways are involved in the recognition and destruction of human erythrocytes during extracorporeal porcine liver perfusion. Chihara et al. have illustrated that the consumption of human platelets by porcine KC is mediated, in part, by CD18 on the surface of the KC [28]. CD18 is known to have a carbohydrate binding site for N-acetyl glucosamine β1-4 N-acetyl glucosamine (βGlcNac) oligosaccharides [29]. Recently, Paris et al has shown that the expression level of βGlcNac

is higher on the surface of human platelets as compared to the expression level on porcine platelets [30]. It is possible that CD18 expressed on the surface of the KC are involved in mediating the destruction of human erythrocytes. It is also possible that human erythrocyte destruction is mediated in part by receptors known to play active roles in immune signaling. For instance, CD47 and SIRPα are known to serve as markers of self and are said to serve as a "don't eat me" signal in the case that autologous cells come into contact with a "self" phagocytic cell [31, 32]. "Self" CD47 causes phosphorylation of SIRPα and ultimately blockade of phagocytosis [33]. However, in the case of interactions across the species barrier as in the setting of xenotransplantation, SIRPα is not phosphorylated and thus, phagocytosis is permitted to proceed [31]. It is likely that CD47 and SIRPα are involved in mediating the anti-erythrocyte response described above. However, given in vitro data from our laboratory showing 1F1 mAb treatment of cultured macrophages reduces the amount of human erythrocyte binding to levels comparable to that of autologous erythrocyte binding, we propose that if the CD47/SIRPα pathway is involved, it acts a as regulator downstream from the initial pSn-mediated binding.

The present study illustrates the involvement of pSn in recognition events leading to the destruction of human erythrocytes during extracorporeal porcine liver perfusion. Although the addition of an anti-pSn mAb did not fully rescue erythrocytes from destruction, the partial blockade of erythrocyte destruction seen in livers treated with anti-pSn mAb suggests that refinements in preventing this recognition pathway will lead to improvement in this potential therapeutic approach. Future studies utilizing livers derived from pSn-deficient pigs would clarify the relative importance of pSn recognition and enable identification of alternative receptor-ligand interactions that may be involved.

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Abbreviation

FHF	Fulminant hepatic failure
pSn	Sialoadhesin
ECLP	Extracorporeal liver perfusion
GTKO	α-1,3 galactosyltransferase knockout
hDAF	human decay accelerating factor
KC	Kupffer cells
hRBC	human red blood cells

mAb	monoclonal antibody
Neu5Ac	N-acetylneuraminic acid

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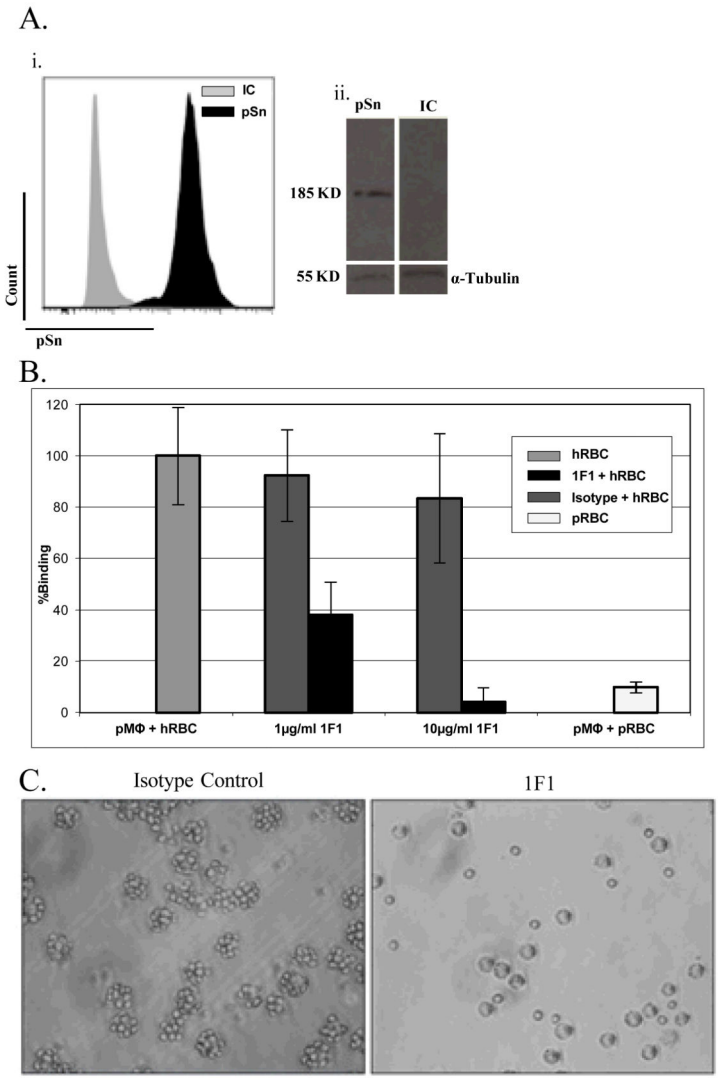
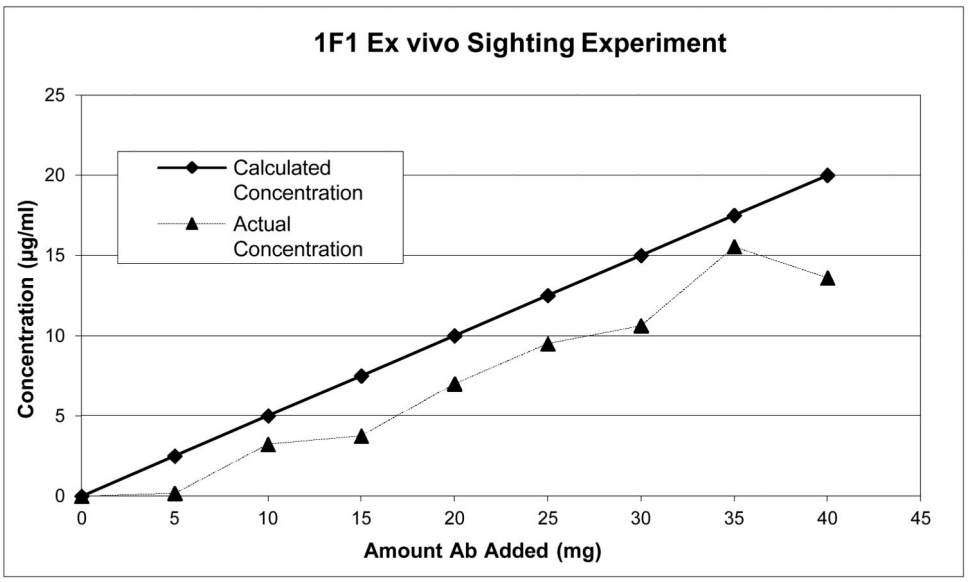


Figure 1. Porcine macrophage mediated binding of human erythrocytes is inhibited by the anti-pSn mAb, 1F1

A: Flow cytometry demonstrated substantial binding of 1F1 mAb to porcine macrophages (pMΦ) as compared with the isotype control (i) (n=3). Immunoblotting confirmed 1F1specificity for pSn (ii). B: Erythrocyte rosetting by porcine macrophages. Human erythrocytes were incubated with porcine macrophages previously treated with either 1F1 mAb (Black) or isotype control Ab (Dark Grey) at a concentration of either 1μg/ml or10μg/ml. In addition, erythrocytes were incubated with pMΦ left untreated (Light Grey). Compared with the isotype control, pretreatment of pMΦ with 1F1 led to significant inhibition of human erythrocyte rosette formation (1μg/ml = p,<0.01 and 10μg/ml = p,<0.001) (n=3). Treatment of porcine macrophages with isotype control antibody did not significantly decrease human erythrocyte binding as compared to untreated pMΦ (1μg/ml = p,NS and 10μg/ml = p,NS) (n=3). For comparison, porcine macrophage binding to porcine erythrocytes was determined (White). Error bars represent standard deviation. C: Phase contrast micrographs of porcine macrophages co-incubated with human erythrocytes.

Porcine macrophages were either treated with 10μg/ml isotype (Left) or 10μg/ml 1F1 mAb (Right).

A.



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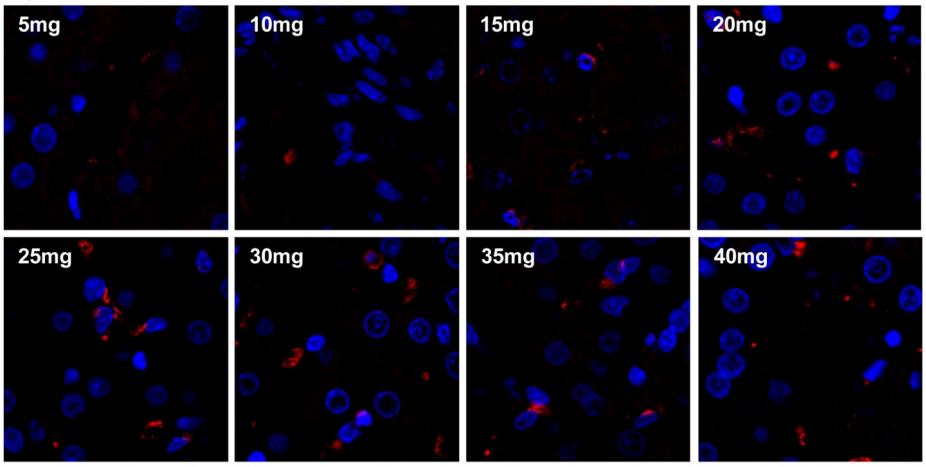

Figure 2. Kinetics of 1F1 mAb in the ex vivo liver perfusion model

1F1 mAb serum concentration and binding in the liver were assessed in an *ex vivo* liver perfusion model. A single porcine liver was perfused with increasing amounts of 1F1 mAb delivered every hour administered in 5 mg boluses. A: During the perfusion, serum samples were collected and serum 1F1 mAb concentration was analyzed with ELISA (circulating 1F1 mAb concentration). Expected 1F1 mAb serum concentration is shown as calculated 1F1 mAb concentration. B: Punch biopsies of the liver were collected throughout the perfusion and analyzed for the presence of 1F1 mAb. Samples were stained with a fluorescently labeled goat anti-mouse IgG_{2a} secondary (Red). Nuclei were stained with DAPI (Blue).

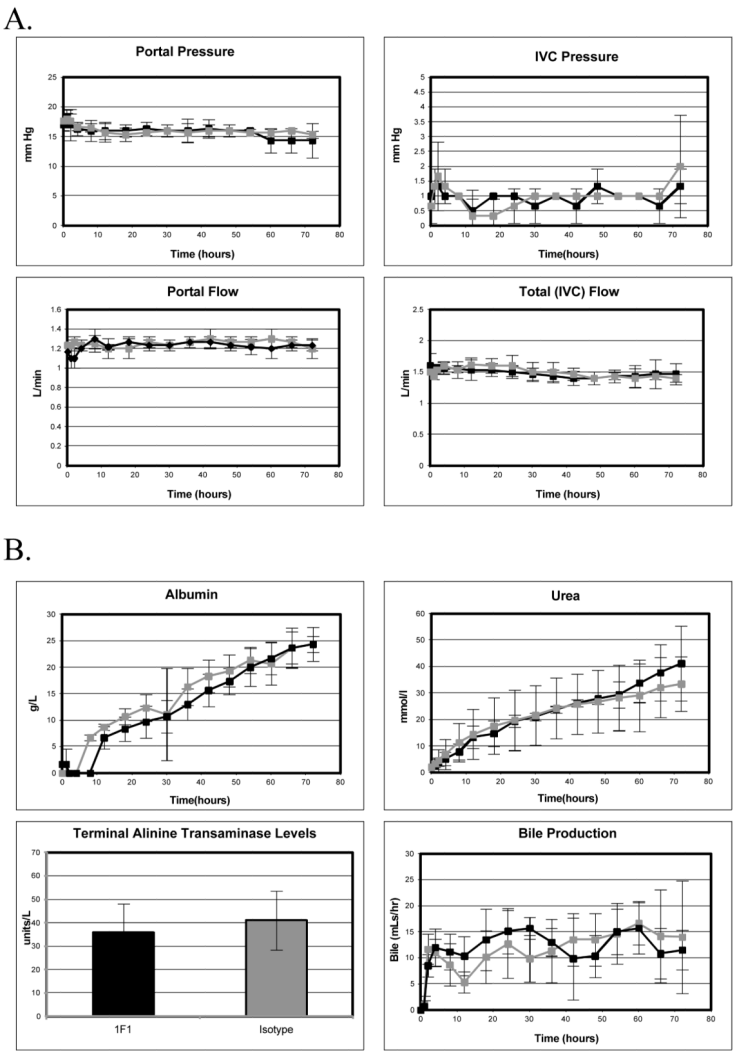

Figure 3. Normal hemodynamic and synthetic function in livers treated with 1F1 mAb

A: Hemodynamic parameters were measured throughout perfusion. Portal vein and inferior vena cava (IVC) (total) flow rate were measured during perfusion of 1F1 mAb (Black) and isotype control Ab (Grey) (p,NS and p,NS respectively). Additionally, portal and IVC pressure were measured throughout the 72 hour perfusion (p,NS and p,NS respectively). (N=3) B: Synthetic function of 1F1 mAb treated (Black) and isotype control Ab treated (Grey) livers was assessed throughout the perfusion by measuring albumin (p,NS), urea (p,NS), and bile production (p,NS). As a marker of hepatocyte injury during extracorporeal perfusion, alanine transaminase levels were assessed at the termination of perfusion and no difference was seen between 1F1 and isotype (p, NS). (N=3) Error bars represent standard deviation.

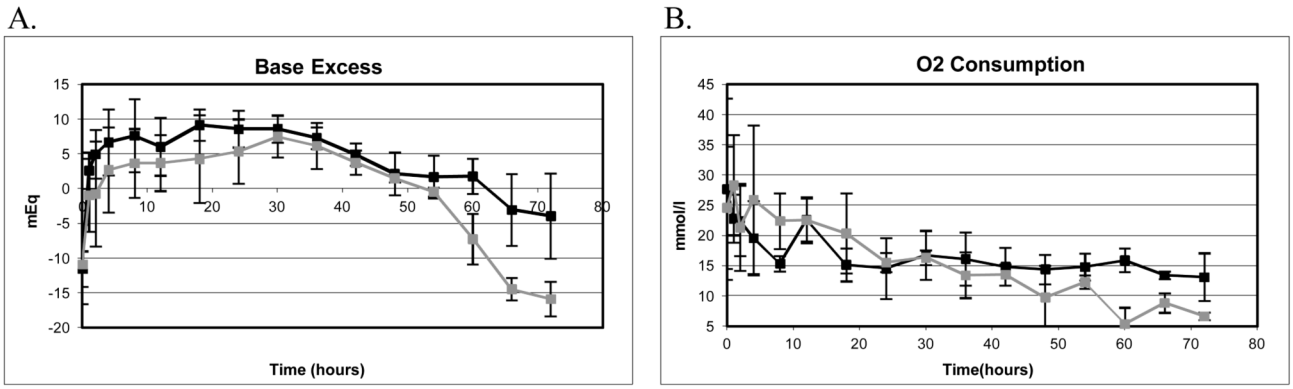

Figure 4. Addition of 1F1 mAb prolongs metabolic function

Metabolic function was measured throughout the perfusion of livers treated with 1F1 mAb (Black) and the isotype control Ab (Grey). Base Excess (A): and oxygen consumption (B): were measured. Significant differences were seen beginning at 48 hours for base excess and 54 hours for oxygen consumption (p,0.003 and p,0.003 respectively, N=3). Error bars represent standard deviation.

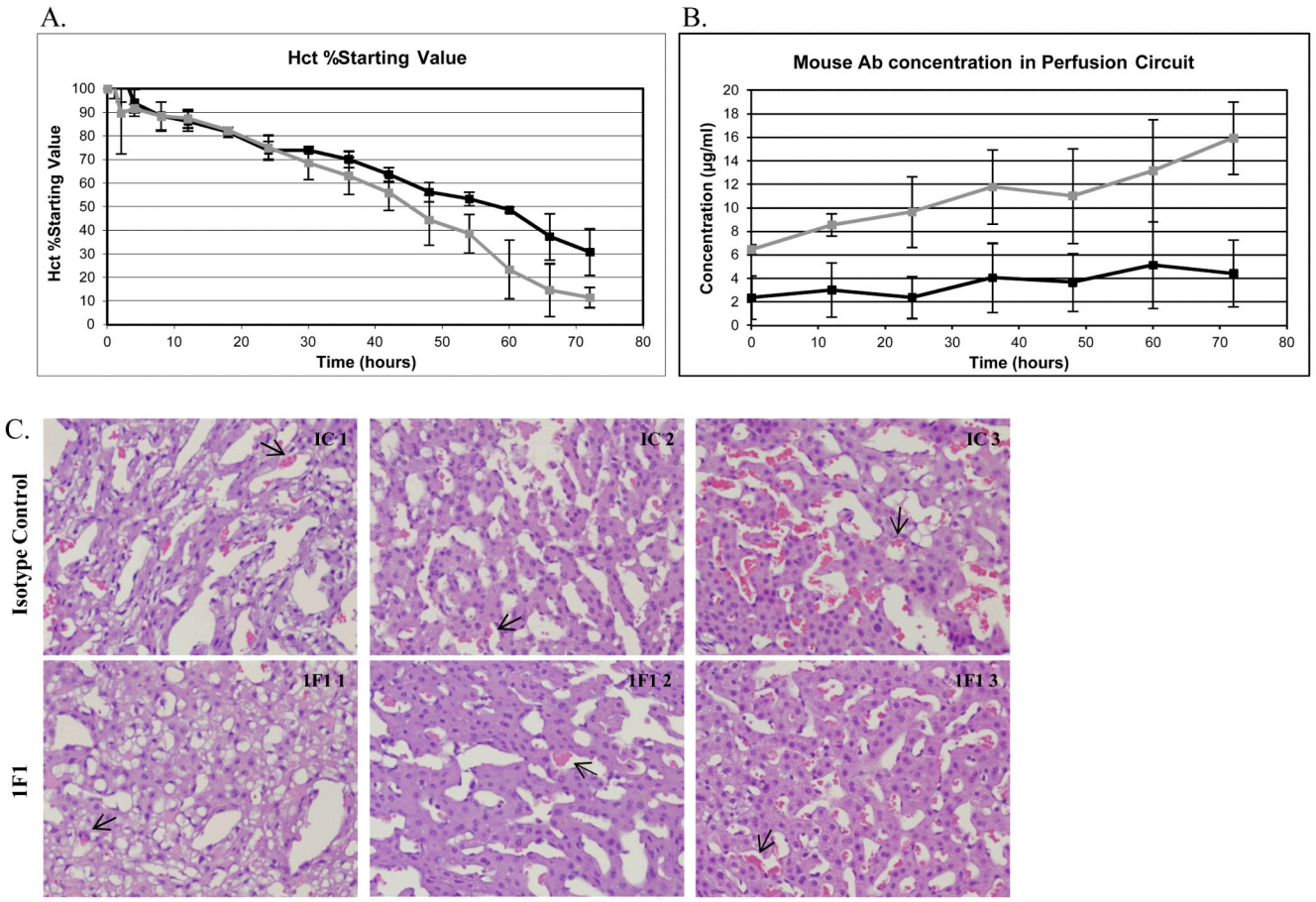

Figure 5. Kupffer cell mediated erythrocyte binding and partial rescue of hematocrit with 1F1 mAb

A: The percentage of intact erythrocytes was measured as % circulating hematocrit, during perfusion of livers treated with 1F1 mAb (Black) or the isotype control Ab (Grey) (p,0.01) (n=3). B: Mouse Ab concentrations in the serum of livers treated with 1F1 mAb (Black) and the isotype control Ab (Grey) were analyzed using ELISA (p,<0.01, n=3). Error bars represent standard deviation. C: Hemotoxylin and Eosin staining was performed on tissue collected at the end of the perfusion from livers treated with 1F1 mAb and the isotype control Ab. Black arrows represent erythrocyte rosetting.

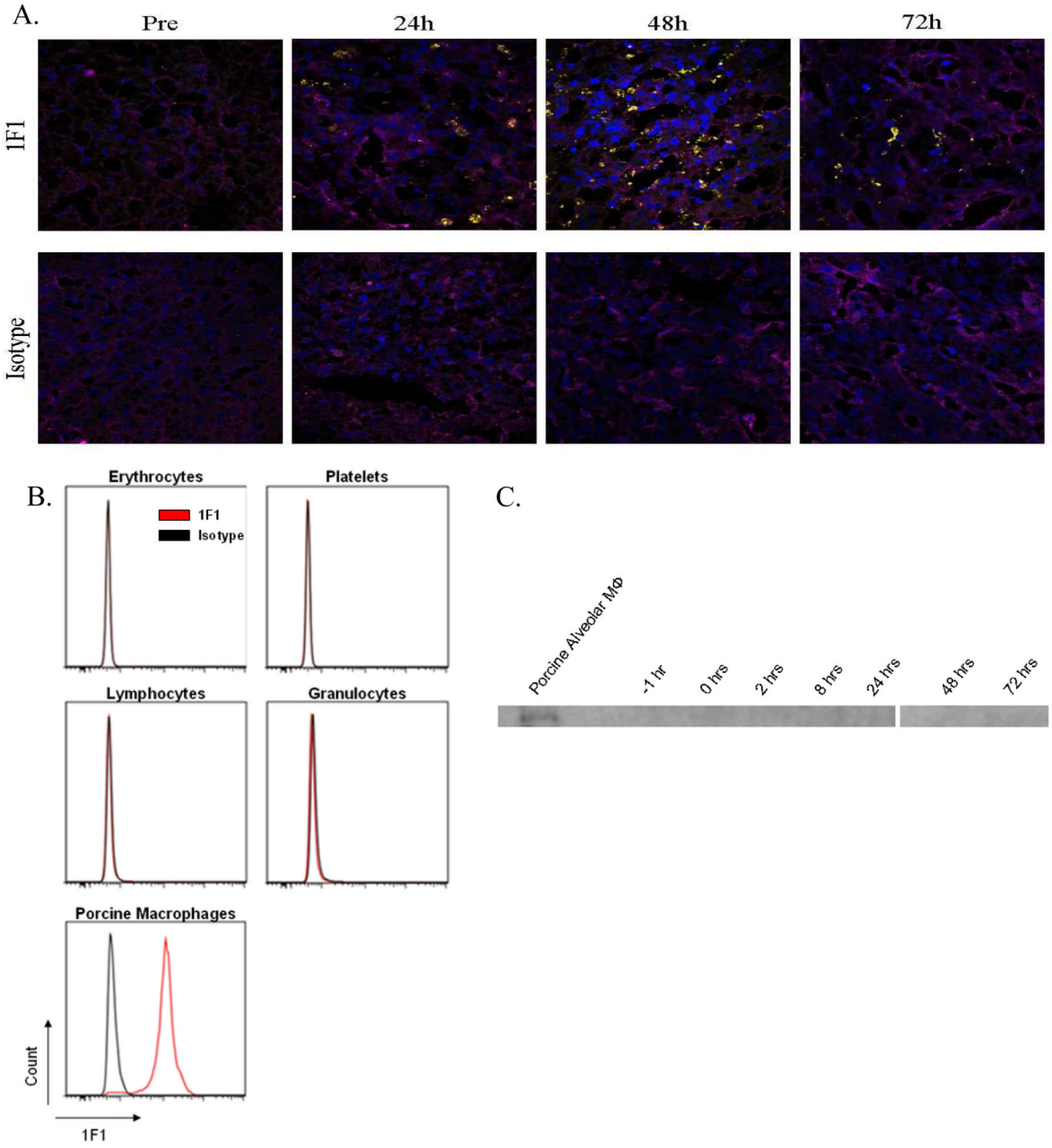

Figure 6. 1F1 mAb localized in the liver

A: Confocal analysis of the presence of mouse Ab, either 1F1 mAb (Top Row) or isotype control Ab (Bottom Row), was detected in the liver by staining fresh frozen biopsies taken at indicated times. Samples were stained with a fluorescently labeled goat anti-mouse IgG_{2a} secondary (Yellow). Endothelial cells were labeled for CD31 (Magenta). Nuclei were stained with DAPI (Blue). B: 1F1 mAb did not bind to human: erythrocytes, platelets, lymphocytes, or granulocytes in the perfusate when labeled with 1F1 mAb (Red) or the isotype control Ab (Black) and analyzed by flow cytometry. Porcine alveolar macrophages were used as a positive control. (n=3) C: 1F1 mAb does not bind soluble pSn in perfusate.

Western blot analysis was performed on serum collected during the perfusion at the times indicated and probed using 1F1 mAb. Porcine macrophage homogenates were used as a positive control.